1.0 ICCVAM EVALUATION AND RECOMMENDATIONS ON ESTROGEN AND ANDROGEN RECEPTOR BINDING AND TRANSCRIPTIONAL ACTIVATION ASSAYS

ICCVAM evaluates the scientific validity of new, revised, and alternative toxicological test methods applicable to Federal agency safety testing requirements, and provides recommendations to Federal agencies about the usefulness and limitations of such methods (P.L. 106-545). In 2000, EPA requested that ICCVAM conduct an independent scientific peer review of the validation status of *in vitro* ER and AR binding and TA assays. This section describes the evaluation completed by ICCVAM in collaboration with NICEATM, and provides ICCVAM's recommendations on these test methods.

1.1 Introduction

In vitro ER and AR binding and TA assays are proposed as part of EPA's EDSP Tier 1 screening battery of *in vitro* and *in vivo* test methods designed to identify substances capable of interacting with the endocrine system. Data generated by these Tier 1 screening assays will be used to make decisions based on a weight-of-evidence approach on whether to conduct Tier 2 testing. With partial support from EPA, NICEATM conducted a comprehensive literature search for relevant publications on these test methods. In addition to this literature search, NICEATM requested through the *FR* (66 *FR* 57: 16278-16279, March 23, 2001) that interested scientists submit published and unpublished data on these test methods for consideration. A draft BRD was prepared for each of the four types of assays (NIEHS 2002a, 2002b, 2002c, 2002d). Each BRD includes:

- a description of the types of test methods used to measure the endpoints of interest and the available data substantiating their scientific validity;
- published and submitted data on substances tested in the test methods being considered;
- an evaluation of the comparative reliability and performance of the test methods being considered;
- test method specific protocols provided by interested scientists;
- a prioritized list of test methods recommended for validation;
- proposed minimum procedural standards for the types of test methods being considered; and
- a list of substances proposed for future validation studies.

The review revealed that no inter- and intra-laboratory validation studies had been conducted on *in vitro* ER or AR binding and TA assays. Therefore, ICCVAM and EPA agreed that an Expert Panel should be convened to evaluate currently available test methods and to recommend future validation efforts. NICEATM, in collaboration with the EDWG, subsequently organized an Expert Panel meeting to evaluate the current status of ER and AR binding and TA assays.

1.1.1 ICCVAM/NICEATM Expert Panel Meeting

The Expert Panel meeting was held on May 21 and 22, 2002, at the Sheraton Imperial Hotel in Research Triangle Park, North Carolina. The 24 members of the Expert Panel (a list of members is provided in the **Acknowledgments** section) reviewed the four draft BRDs, assessed the current validation status of the four types of *in vitro* assays described in **Section**s **1.1.1.1** through **1.1.1.4**, and developed recommendations (see **Appendix A**) on:

- test methods that should be considered for further evaluation in validation studies and their relative priority;
- the adequacy of the proposed minimum procedural standards for each of the four types of test methods;
- the adequacy of available protocols for test methods recommended for validation studies; and
- the adequacy and appropriateness of the substances recommended for use in the validation studies.

1.1.1.1 *In Vitro* ER Binding Assays

The Expert Panel reviewed 14 different *in vitro* ER binding assays in which 638 different substances had been tested at least once in one or more of the test methods (NIEHS 2002a). The sources of the ER for the different test methods included:

- cytosol prepared from MCF-7 cells, a cell line derived from human breast cancer adenocarcinoma cells;
- cytosol from the uteri of mice, rats, and rabbits;
- intact MCF-7 cells;
- purified recombinant human ERα and ERβ; and
- fusion proteins between glutathione-S-transferase and the binding domains of the human ERα, and ER from mouse, chicken, anole (a reptile), and rainbow trout.

1.1.1.2 *In Vitro* ER TA Assays

The Expert Panel reviewed 95 different ER TA assays (73 mammalian cell and 13 yeast strain reporter gene assays, and 9 mammalian cell proliferation assays) in which 698 different substances had been tested at least once in one or more of the test methods. The source of the ER included:

- unspecified ERs from human, mouse, and rat; and
- ERα and ERβ subtypes found endogenously, or transiently or stably transfected into various cell lines.

The reporter genes used in these test methods included:

- luciferase and chloramphenicol acetyltransferase in the mammalian cell line assays; and
- β-galactosidase in the yeast assays.

1.1.1.3 *In Vitro* AR Binding Assays

The Expert Panel reviewed 11 different *in vitro* AR binding assays in which 108 different substances had been tested at least once in one or more assays. The sources of AR used in these test methods included:

- cytosol from calf uteri, rat epididymes, rat prostate glands, and MCF-7 cells;
- rat epididymal nuclear fraction;
- COS-1 cells transiently transfected with human AR;
- human genital fibroblasts with an endogenous AR;
- LNCaP cells with an endogenous mutant AR; and
- semipurified recombinant human AR.

1.1.1.4 *In Vitro* AR TA Assays

The Expert Panel reviewed 17 different AR TA assays (15 mammalian cell and 1 yeast reporter gene assays, and 1 mammalian cell proliferation assay), in which 145 different substances had been tested at least once in one or more of the assays. The source of the AR used in these test methods included ARs from human, mouse, and rat.

The reporter genes used in the test methods included:

- luciferase and chloramphenicol acetyltransferase in the mammalian cell line assays; and
- β-galactosidase in the yeast assay.

1.1.1.5 Final Report of the Expert Panel

The Expert Panel's conclusions and recommendations on each type of test method are provided in its final report (**Appendix A**). The four draft BRDs were subsequently revised to incorporate changes and corrections recommended by the Expert Panel (see **Section 1.1.1**). Electronic copies of the final BRDs are available on the ICCVAM/NICEATM website *http://iccvam.niehs.nih.gov/methods/endocrine.htm*.

1.2 ICCVAM Proposed Substances for Validation of *In Vitro* Endocrine Disruptor Assays

To facilitate future validation efforts and the comparison of performance among different test methods and protocols, the EDWG, NICEATM, and ICCVAM drafted a list of 122 proposed substances to be used in future validation studies for each of the four types of assays. This list incorporated:

- substances proposed in the four BRDs and endorsed by the Expert Panel;
- other substances recommended by the Expert Panel;
- substances proposed by EPA for validation of *in vitro* ER and AR binding assays and by EPA and the Organisation for Economic Co-operation and Development (OECD) for validation of *in vivo* endocrine disruptor assays (a list of these substances was compiled by Mr. James Kariya of the EPA and presented at the March 2002 meeting of the EPA Endocrine Disruptor Methods Validation Subcommittee [EDMVS]);
- substances to address the Expert Panel's recommendation that the list contain at least 25% negative substances in order to adequately characterize test method specificity; and
- the Expert Panel's recommendation that, for a specific receptor (ER or AR), the same substances should be tested in both binding and TA agonism and antagonism assays.

Subsequently, this draft list of 122 substances was reduced to a draft list of 78 proposed substances. Public comments on this draft list of proposed substances are provided in **Appendix F** and are discussed in **Section 1.3**. The substance selection criteria and the process used to develop the final proposed list of substances are described in **Section 2.0**.

To comprehensively assess the usefulness of binding and TA assays as individual components of the Tier 1 screening battery that will be used to prioritize substances for Tier 2 testing, and to facilitate development of more predictive *in vitro* endocrine disruptor assays, all 78 substances should be tested in the four types of assays. However, this list contains a relatively high proportion of substances, about 49% and 57%, which are anticipated to be negative in *in vitro* ER- and AR-based assays, respectively (see **Section 2.0**; **Expert Panel Report, Appendix A**). As only 25% negative substances are needed to adequately assess test method specificity, characterizing the activity of all 78 substances in *in vitro* ER and AR binding and TA assays might not be essential. Therefore, the EDWG and ICCVAM identified a list of 53 substances for ER-based assays and 44 substances for AR-based assays that should be used, at a minimum, during the validation of these test methods. These lists are discussed in **Sections 3.0** through **6.0**.

1.3 Public Comments

NICEATM announced in a *FR* notice (67 *FR* 204: 64902-64903, October 22, 2002) the availability of the Expert Panel's report and the EDWG's draft proposed list of substances for validation studies, and requested public comment. The final versions of the four BRDs and the summary minutes of the Expert Panel meeting (**Appendix D**) were

made available on the ICCVAM/NICEATM website *http://iccvam.niehs.nih.gov/methods/endocrine.htm*. Five public comments were received; these are briefly discussed in this section. The original comments are provided in **Appendix F**.

1.3.1 Comments Regarding the Suitability of Transcriptional Activation Assays Being Developed for Commercial Testing

Dr. Mitsuru Iida (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) submitted comments which focused on the ability of Otsuka's *in vitro* TA assays, under development for commercial testing, to meet the recommendations of the Expert Panel for such test methods. Data and information were provided to support this position, including:

- The Otsuka AR-Ecoscreen™ assay uses a stably transfected cell line, which contains an androgen response element for which the AR has high affinity, and low levels of the glucocorticoid receptor.
- The Otsuka method for transfection of the reporter plasmid differs from the approach recommended by the Expert Panel in that the plasmid and the transfection reagent are added directly to the cells in the medium in which they are plated. This approach is reported as being superior to the adenovirus-based method of transduction recommended by the Expert Panel.
- The Otsuka AR-Ecoscreen™ can detect weak agonists and antagonists.
- The intra-assay coefficient of variation (CV) is 3.2% for studies using the stably transfected cell line and 5.9% for studies using the transiently transfected cell line. The corresponding inter-assay CVs are 8-14% and 16-22%. These CVs are reported as being less than those determined for the corresponding adenoviral transduction-based assay.
- An efficient internal monitor of cytotoxicity is included in each study.
- Corresponding ER TA assays with equal reliability have been developed.
- The test methods can be reliably applied at this time.

ICCVAM recognizes that the *in vitro* test methods developed by Otsuka might have merit, and suggests that Otsuka consider the recommendations contained in this report regarding minimum procedural standards and the substances proposed for validation studies, as well as the ICCVAM Submission Guidelines[1]. Following the completion of appropriate validation studies, the test methods can be submitted to ICCVAM for evaluation.

1.3.2 General Comments from the American Chemistry Council

Comments were submitted on behalf of the American Chemistry Council (ACC) by Dr. Richard Becker (Arlington, Virginia) regarding the Expert Panel's Report and the list of proposed substances for validation studies. With respect to the binding and TA assays, the comments addressed the following points:

- EPA is obligated to validate a binding assay and a TA assay for AR and ER ligands if it intends to require submission of data from such assays as part of the EDSP. However, it is important to recognize that extensive use of any particular test method in basic academic research does not *de facto* validate its use for regulatory toxicity testing.
- There is an urgent need to validate a single technique for each type of assay. As noted in the Expert Panel report, there currently exists significant variability in techniques

[1] Available at *http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm*.

and results. Furthermore, interlaboratory variability, sensitivity, reproducibility, and precision have not been sufficiently evaluated. The use of recombinant receptor proteins to reduce animal use and to more fully standardize components of the test method should be encouraged.

- EPA needs to address the patent restriction issues. It is essential that the test methods required for regulatory programs are widely available and that the regulated community is not put at risk of violating patents in order to comply with screening and testing requirements.

Comments submitted regarding the proposed list of substances included the following:

- Criteria need to be developed to select substances for validation efforts.
- Substances must be appropriately qualified and characterized.
- Each proposed substance must be appropriately referenced.
- The draft list needs to be reviewed and appropriately referenced, and any errors or omissions corrected.

ICCVAM agrees with the constructive comments provided by the ACC. Comments relevant to EPA will be brought to its attention when this report is forwarded to Federal agencies. The list of proposed substances has been revised with due consideration of the comments made. The selection criteria used to develop the final list of substances are provided in **Section 2.0**.

1.3.3 Chemical Abstracts Service Registry Number for Commercially Available Nonylphenol

A comment was provided by Dr. Barbara Losey of the Alkylphenols and Ethoxylates Research Council (AERC; Washington, District of Columbia) regarding the form of nonylphenol included in the list of proposed substances for validation studies. This nonylphenol (*p*-n-nonylphenol; Chemical Abstracts Service Registry Number [CASRN] 104-40-5) consists of a linear alkyl chain and is not representative of the commercial forms of nonylphenol. Commercial synthesis results in a mixture of various branched nonylphenol isomers represented by the CASRN 84852-15-3 rather than the production of one substance with a discrete chemical structure. The AERC believes that the commercial product is more relevant to human exposure and also the substance most frequently tested in *in vivo* endocrine disruptor studies.

Based on an assessment of the data in the BRDs, information on the specific form of nonylphenol tested in *in vitro* ER and AR binding and TA assays (as defined by the inclusion of a CASRN in the report) was provided for only 1 of 29 studies. In this single study, the commercial form of nonylphenol (CASRN 84852-15-3) was tested. However, while ICCVAM recognizes that *p*-n-nonylphenol is not a commercially relevant substance, this isomer is recommended for validation studies because its chemical structure is uniform. Samples of the commercial product would be expected to vary considerably in the ratio of various isomers, and this variability in chemical structure might contribute to increased variability in response across test methods. In post-validation studies, the form of the substance most relevant to human exposure should be tested.

1.3.4 Scintillation Proximity Assay

Information was provided by Mr. Mike Scully (Amersham Biosciences, Cardiff, United Kingdom) about a scintillation proximity assay that measures the binding of a ligand to a receptor which is bound to a glass bead coated with a scintillant. Mr. Scully stated that this method eliminates washing steps and is fully amenable to automation. He stated also that

this method has applicability to the binding of ligands to ER and AR proteins and thus should be considered for future development efforts. References were provided on scintillation proximity assays, including one application to ER binding.

ICCVAM recognizes that the scintillation proximity assay developed by Amersham Biosciences might have merit. ICCVAM suggests that Amersham Biosciences consider the recommendations contained in this report regarding minimum procedural standards and the substances proposed for validation studies, as well as the ICCVAM Submission Guidelines[2] if the company decides to submit their assay for evaluation to ICCVAM.

1.3.5 Response of Atrazine in ER and AR Binding and TA Assays

In the list of proposed substances for validation studies, the "anticipated *in vitro* response" for atrazine was that it would bind weakly in both ER and AR binding assays but would be negative in ER and AR TA assays. Dr. Charles Breckenridge (Syngenta Crop Protection, Inc., Greensboro, North Carolina) submitted a comment in which he noted that, based on the available data, it would be more appropriate to classify atrazine as negative and unknown for ER- and AR-based assays, respectively.

ICCVAM has revised the substance lists to categorize atrazine and other substances that were positive in 50% or fewer of the reported studies, as "presumed positives" for the *in vitro* endocrine disruptor assay of interest. This classification is used because erroneous positive studies are probably less likely to occur than erroneous negative studies due to the nature of binding assays and the protocols generally used. While this presumed positive classification is subjective for substances that test negative in the majority of tests conducted, it is anticipated that testing these substances will provide critical information on the comparative sensitivity and reliability of different *in vitro* endocrine disruptor assays.

[2]Available at *http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm*.

1.4 ICCVAM Recommendations

ICCVAM reviewed the Expert Panel's report (provided in **Appendix A**), and concurs with their conclusions and recommendations. For convenience to the reader, the major recommendations and conclusions are summarized in this section. More detailed information and discussion can be found in the Expert Panel's report. Other important considerations and additional recommendations from ICCVAM are provided in **Section 1.4.5**.

1.4.1 *In Vitro* ER Binding Assays

- Recombinant rat or human ERs (α and β subtypes) should be given the highest priority for further test method standardization, prevalidation, and validation. Recombinant receptors are superior to crude cytosolic preparations because they can be prepared and distributed as standardized products with significantly less contamination. This will result in greater reproducibility and facilitate comparison of results across laboratories. To screen for possible ecological effects, recombinant receptors from wildlife are considered to be potentially more relevant and their use should be evaluated.
- Although it would be advantageous to use nonradioactive methods such as fluorescent polarization to assess ER binding, this method has not been widely used and specialized equipment is required. However, once a test method using recombinant ER proteins has been validated, there should be an effort to

optimize a fluorescence-based method to replace the use of radioactivity.

- *In vitro* ER binding assay protocols should be standardized to incorporate the recommended minimum procedural standards (see **Section 3.1**). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the test method should be conducted.
- Proposed *in vitro* ER binding test methods should be evaluated in validation studies using, at a minimum, the 53 substances listed in **Section 3.2**. This list includes substances that cover a range of activities, from negative to weakly positive to strongly positive, with 40 (75%) positive and presumed positive and 13 (25%) negative and presumed negative substances. The list also represents a wide range of relevant chemical and product classes (see **Section 2.0**). Following validation studies using the 53 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of the screening test battery for prioritizing substances for subsequent *in vivo* studies.

1.4.2 *In Vitro* ER TA Assays

- A comparative study should be conducted to determine whether transiently or stably transfected cell lines are more appropriate for a routine test system. Transiently transfected systems generally have a higher level of responsiveness, while stably transfected cell lines have a lower level of responsiveness but are generally more amenable to high-throughput screening. Such a study should use cell lines with the same ER reporter gene constructs. A third cell line expressing an endogenous ER and transfected with the same reporter construct should be included in this study.
- *In vitro* ER TA assay protocols should be standardized to incorporate the recommended minimum procedural standards (see **Section 4.1**). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the test method should be performed.
- To facilitate the comparison of *in vitro* ER-based assays, the same minimum list of 53 substances (provided in **Section 4.2**) recommended for ER binding assays should be used in the validation of *in vitro* ER TA agonist and antagonist assays. For ER TA agonism and antagonism assays, 34 (64%) and 11 (21%) of the substances, respectively, are reported to be positive or presumed positive, and 19 (36%) and 42 (79%) of the substances, respectively, are presumed negative. Following validation studies using the 53 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of a screening test battery for prioritizing substances for subsequent *in vivo* studies.

1.4.3 *In Vitro* AR Binding Assays

- A recombinant protein should be used as the source of the AR. Recombinant receptors are superior to crude cytosolic preparations because the recombinant protein can be standardized, which contributes to improved quality control and comparison of results across laboratories. Thus, the highest priority for future research and development efforts should

be given to the development of a test method using a recombinant full-length AR protein. Patents on the AR protein have hindered development of this assay.

- *In vitro* AR binding assay protocols should be standardized to incorporate the recommended minimum procedural standards (see **Section 5.1**). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.
- Proposed *in vitro* AR binding assays should be evaluated in validation studies using, at a minimum, the 44 substances listed in **Section 5.2**. This list consists of 33 (75%) positive and presumed positive substances and 11 (25%) presumed negative substances for AR binding. Following validation studies using the 44 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an *in vitro* test battery for prioritizing substances for subsequent *in vivo* studies.

1.4.4 *In Vitro* AR TA Assays

- None of the *in vitro* AR TA assays reviewed by the Expert Panel were considered optimal for assessing AR agonist and antagonist activities. The highest priority for future efforts should be a cell line containing an endogenous AR that is transduced with an adenovirus containing a reporter vector that shows high specificity for the AR. The chosen cell line should not respond to, or have minimal response levels for, the glucocorticoid and progesterone receptors. Because of patent restrictions, it may be necessary that a cell line with an endogenous AR be used for validation. Transduction of a reporter construct in a virus particle is more efficient and reproducible than transfection of a construct.
- *In vitro* AR TA assay protocols should be standardized to incorporate the recommended minimum procedural standards (see **Section 6.1**). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.
- To facilitate *in vitro* AR-based assay comparisons, the same minimum list of 44 substances (provided in **Section 6.2**) recommended for *in vitro* AR binding assays should be used in the validation of *in vitro* AR TA agonist and antagonist assays. For AR TA agonism and antagonism assays, 20 (45%) and 20 (45%) of the substances, respectively, are reported to be positive or presumed positive, and 24 (55%) and 24 (55%) of the substances, respectively, are presumed negative. Following validation studies using the 44 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an screening test battery for prioritizing substances for subsequent *in vivo* studies.

1.4.5 Other Recommendations

ICCVAM agrees with the Expert Panel that the development and validation of *in vitro* ER and AR binding and TA assays should emphasize the use of recombinant-derived proteins. Based on current knowledge and experience, it appears that continuing to use animal-derived ER or AR in *in vitro* endocrine disruptor assays requires scientific justification. The

advantages of using recombinant-derived receptors for binding assays include:

- Standardized recombinant protein can be prepared and used by multiple laboratories, which will contribute to improved inter- and intra-laboratory reproducibility and an enhanced ability to compare results across laboratories.
- Recombinant-derived receptors avoids the disadvantages of animal-derived receptors, which include:
  - The receptors, particularly the ARs, are unstable in tissue extracts.
  - The cytosolic extracts contain many proteins, including other endogenous steroid receptors that can interfere with the performance of the assay.
  - Animals have to undergo surgery before isolation of the tissue of interest. For AR binding assays, males are castrated, and, for ER binding assays, females undergo an ovariectomy before removal of the requisite tissues and isolation of the respective receptors.
  - Animals need to be killed to obtain either the uterus (ER binding) or prostate (AR binding) glands.
- The inclusion of a metabolic activation system in *in vitro* ER and AR binding and TA assays is not recommended at this time, as the type of metabolic activation system developed will depend on which *in vitro* assays are selected. Available information on the metabolism of the validation substances should be compiled, including the degree to which metabolism is known to alter estrogenic and androgenic activity *in vivo*. Once the importance of metabolic activation in the ability of substances to disrupt endocrine function has been demonstrated, and valid *in vitro* ER and AR binding and TA assays have been identified, appropriate methods for including metabolic activation in the assays can be developed and validated.
- The current analyses for making statistical inferences with *in vitro* endocrine disruptor data require more detailed research and study. Appropriate prevalidation studies should be conducted to generate data necessary for biostatisticians to develop appropriate statistical methods for analyzing binding and TA agonist and antagonist assay data.
- Although these *in vitro* endocrine disruptor assays are proposed as components of a screening test battery where the results will be used in making weight-of-evidence decisions, the predictive value of these *in vitro* assays for estimating *in vivo* responses should be determined. To facilitate this determination, ICCVAM recommends that all 78 substances (see **Section 2.0**) should be evaluated in each *in vitro* assay. It is only through this effort that the performance of the *in vitro* assays for predicting responses in animals can be evaluated and decisions made as to whether and how *in vitro* assays can reduce or replace animal use. Such data will also be needed to determine the usefulness of the *in vitro* battery for prioritizing substances for further testing.
- A centralized repository of the 78 substances with verified purity should be organized to facilitate future validation studies. The purpose of this repository is to provide a source of coded samples, of known purity, for validation studies. This approach would greatly enhance evaluation of the comparative reliability and performance of different versions of *in vitro* ER and AR binding and TA assays.
- Federal agencies are encouraged to support research and development of new technologies (e.g., genomics) that may provide more accurate assessments and/or advantages in terms of time and cost.

1.5 Other Considerations

- The Panel recommended that appropriate government agencies investigate the status of patents and licenses pertinent to the use of the human and rat AR and provide guidance as to how the scientific community should proceed with the development of *in vitro* AR assays.
- Although there is more information and data on ER binding studies with human ERα and ERβ than the equivalent receptors from rats, it might be more appropriate for the rat ERα or ERβ to be used for validation than the human receptors. This is because the rat is being used as the mammalian species of choice for *in vivo* Tier 1 and Tier 2 assays. Because the rat ERα has been isolated from the uterus and the ERβ from the prostate, the rat ERα would likely be the most appropriate receptor for ER binding studies (Kuiper et al., 1996). A study should be conducted to compare the responsiveness of the ERα from the rat to the ERα from humans in order to assess potential differences in the binding capacities of the receptor from the two species.